Early morning run-training results in enhanced endurance performance adaptations in mice.

Running title: Time-of-day run training and performance.

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Key points summary:

- Time-of-day differences in exercise performance are well established in the literature. We observe that after 6 weeks of endurance exercise training there is no longer a time-of-day specific difference in endurance performance.

- Consistent endurance training performed in morning (ZT13) results in a greater performance increase compared to exercise training during the afternoon (ZT22).

- Removal of time-of-day differences in performance through exercise training is strongly associated with significant phase shifts (~5 hour advance) of the muscle clock.

Abstract

Time-of-day differences in acute exercise performance in mice are well established with late active phase (afternoon) runners exhibiting significantly greater endurance performance compared to early active phase (morning) runners. In this study, we asked if performance adaptations would be different when training for 6 weeks at two different times of day, and if this corresponds to steady state changes in the phase of peripheral tissue clocks. To address these questions, we endurance trained female PER2::Luciferase mice, at the same relative workload, either in the morning, at ZT13, or in the afternoon, at ZT22. Then, after training, we recorded luminescence from tissues of PER2::Luciferase mice to report timing of tissue clocks in several peripheral tissues. After 6 weeks, we found that both groups exhibited significant improvements in maximal endurance capacity (total treadmill work)($p < 0.0001$), but the morning runners exhibited an enhanced rate of adaptation as there was no detectable difference in maximal endurance capacity ($p = 0.2182$) between the morning and afternoon runners. In addition, morning and afternoon runners exhibited divergent clock phase shifts with a significant 5-hour phase advance in the EDL ($p < 0.0001$) and soleus ($p < 0.0001$) of morning runners, but a phase delay in the EDL ($p < 0.0001$) and Soleus ($p < 0.0001$) of afternoon runners. Therefore, our data demonstrate that morning training enhances endurance adaptations compared to afternoon training in mice, and we suggest this is due to phase advancement of muscle clocks to better align metabolism with exercise performance.

Introduction

Time-of-day differences in endurance exercise performance have been reported in both humans (Chtourou & Souissi, 2012; Souissi *et al.*, 2002; van Moorsel *et al.*, 2016; Gemmink *et al.*, 2023) and rodents (Wolff & Esser, 2012; Ezagouri *et al.*, 2019; Adamovich *et al.*, 2021*a*; Maier *et al.*, 2022). In mice, endurance performance is significantly greater when tested later in the active period, and this is consistent with studies of humans showing increased endurance performance in the afternoon. Recent studies have demonstrated that endurance performance is circadian clock controlled in mice as different genetic mouse models of clock disruption do not exhibit time-of-day exercise performance (Adamovich *et al.*, 2021*a*; Xin *et al.*, 2023*a*). Analysis of tissue glycogen levels suggested liver glycogen stores, linked to feeding, were contributors to the differential time-of-day endurance however there is still very little understood.

The circadian clock mechanism is an evolutionarily conserved transcription-translational feedback mechanism that exists in virtually all cells in the body. The core clock transcription factors *Bmal1* and *Clock* regulate expression of *Period1/2* and *Cryptochrome1/2* genes and their protein products feedback and repress their own expression by inhibiting *Bmal1* and *Clock* transcriptional activity (Ko & Takahashi, 2006; McCarthy *et al.*, 2007; Golombek & Rosenstein, 2010; Mohawk *et al.*, 2012; Partch *et al.*, 2014). This cycle takes ~24 h, and the phase of the clock, defined by the peak expression of core clock factors, is modifiable by environmental time cues, known as zeitgebers. For example, light is a zeitgeber which can adjust the phase of the central circadian clock within the brain. In skeletal muscle, the phase of the clock mechanism can be modified by muscle contractions and exercise (Golombek & Rosenstein, 2010; Wright *et al.*, 2013). This becomes important

for physiological systems as the clock mechanism regulates a daily program of gene expression, termed clock output, that support a temporal pattern of cell physiology (Panda, 2016). Thus, if the clock mechanism in muscle shifts circadian phase, then the downstream clock controlled gene expression program would be shifted, affecting the temporal pattern of physiological outcomes.

Herein, we tested whether 6 weeks of time-of-day run training at the same relative intensity would alter the early active phase (morning) vs. late active phase (afternoon) endurance performance in mice. Our results demonstrate that mice trained in the morning exhibited a greater adaptation in performance compared to those trained in the afternoon. We found that 6 weeks, but not 3 weeks, of training was sufficient to result in the same maximum endurance performance between the morning vs. afternoon runners. We did not detect differences in resting levels of muscle or liver glycogen after training. However, we did determine that the circadian clock mechanism in the skeletal muscles of the morning runners had shifted in phase to ~ 5hrs earlier compared to the sedentary control mice. In contrast, no clocks within white fat, lung, or the central clock shifted in phase in the morning runners. From these findings, we propose that the enhanced training adaptations of the morning runners are linked to the significant shift in the phase of the muscle clocks, which may temporally align time-of-day skeletal muscle oxidative capacity with the times of exercise.

Methods and Materials

Ethical approval

All animal procedures in this study were conducted in accordance with the guidelines of the University of Florida for the care and use of laboratory animals (IACUC #201809136). The use of animals for exercise protocols was in accordance with guidelines established by the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Animals

Eighteen female PERIOD2::LUCIFERASE (PER2::LUC) mice (Yoo *et al.*, 2004) aged 5 months (22 ± 1 g body weight) were bred in-house from mice originally received as a gift from Dr. Joseph Takahashi. Previous data from our laboratory reported no sex-specific effects of exercise training on muscle circadian PER2::LUC phase (Wolff & Esser, 2012), so female mice were selected, as they are known to run more than their male counterparts (Rosenfeld, 2017). Mice were initially housed (12hr light:12hr darkness; ZT0 = time of lights

on/ rest phase, ZT12 = time of lights off/ active phase) in a controlled climate (23 ± 1.5 °C, 58 ± 5.8 % relative humidity) and had *ad libitum* access to water and standard rodent chow (Envigo Teklad 2918, Indianapolis, IN, USA). Mice were then moved to single housing with the same light, climate, and nutritional conditions prior to starting the experimental protocol. All experiments took place during the dark/ active phase, and all treadmill testing and training took place in the dark, under red light, on a Panlab treadmill (Harvard Apparatus, Holliston, MA). All mice were anaesthetized using isoflurane and euthanized by cervical dislocation under red light, ~ 3 days after the last bout of exercise training at ZT17. All tissues were collected at the same time-of-day and included extensor digitorum longus (EDL) and soleus muscles (SOL), along with the suprachiasmatic nucleus (SCN), lungs and white adipose tissue (WAT) which were used for real-time bioluminescence recording. The gastrocnemius and liver were isolated and cleaned of fat and connective tissue then frozen in liquid nitrogen and stored at -80°C pending further analysis.

Maximal Endurance Capacity Testing

Each maximal endurance capacity testing session was performed, similar to that described in (Maier *et al.*, 2022). Briefly, animals began at a speed of 10 cm/s at 10° incline for a 5 min warm-up period. The incline was increased to 15° and the treadmill speed was increased by 3 cm/s every 2 min until exhaustion. The treadmill was operated with the electrical shock grid turned off and sponges were placed at the back of the treadmill to reduce the risk of injury. Mice were deemed exhausted when they remained in contact with the sponge >10 s and could not be encouraged to continue by several air puffs. All mice were tested at the time which corresponded to their group (i.e., either ZT13 or ZT22). The sedentary control group (n = 6) were split so that half (n = 3) were handled at ZT13 in the same manner as the morning training group, and the other half were handled at ZT22 identically to the afternoon training group. This involved being moved from the housing suite into the treadmill room for the duration of each exercise session, here they maintained a sedentary state in their cages and positioned next to the treadmill.

Maximal Endurance Testing Schedule

Initially, mice were randomized into two groups for pilot maximal endurance capacity testing, a morning group (ZT13, n = 9) and an afternoon group (ZT22, n = 9). This was to confirm that there were measurable time-of-day differences in exercise capacity. Immediately

prior to the pre-training trial, mice were subjected to three days of forced treadmill familiarization as in previously described methods (Sato *et al.*, 2019). Briefly, during the first day of familiarization the speed of the treadmill was set to 10 cm/s for 5 min at 0° incline. The incline was then adjusted to 5° and speed was ramped up by 2 cm/s every 2 min up to 20 cm/s. The second day consisted of an increase in speed every 3 min by 3 cm/s from 10 cm/s to 24 cm/s at an incline of 10°. The final familiarization session was the same as the second day but performed on a 15° incline. On the day after the third familiarization session, maximal endurance capacity testing was performed. This was followed by a 10 day washout period where mice where acclimated to new housing. Mice were continuously monitored for daily activity using wireless, infrared activity monitoring (Actimetrics, Wilmette, IL, USA; analyzed using ClockLab software).

Mice were then re-randomized into three experimental groups where they remained for the 6-week time-of-day training: i) training in the morning at ZT13 (n = 6), ii) training in the afternoon at ZT22 (n = 6), or iii) sedentary control (CON, n = 6). These groupings denoted the time-of-day in which testing and training occurred. Similar to the pilot testing the CON group was split so that half (n = 3) were handled at the same time as the morning group, and the other half were handled with the afternoon group. This involved being moved from the housing suite into the treadmill room for the duration of each exercise session, where they were maintained sedentary in their cages and positioned next to the treadmill. Mice had their food removed 1 hour prior to exercise testing and were assessed for their maximal endurance capacity before, after 3 weeks, and after 6 weeks of time-of-day training. Endurance capacity testing conducted after 3 weeks was used to scale exercise intensity to account for training adaptations and assess a time course for improvements.

Endurance Training Program

The training program consisted of 5 exercise bouts per week, over the 6 weeks of training (30 individual exercise bouts), all bouts were consistently performed at the assigned time-of-day training group times (i.e., ZT13 or ZT22). Each training bout consisted of 1 hour of treadmill running at a consistent slope of 15° and a speed corresponding to 70 % of work done (Equation 1) during maximal endurance testing, calculated according to equations from (Avila *et al.*, 2017).

Equation 1. work done = $M \cdot D \cdot \sin\theta$

Were M is the mass (g) of the animal, D is the distance (m) ran in the maximal capacity test and θ is the slope (°) of the treadmill. Work done is measured in arbitrary units (Avila *et al.*, 2017).

Blood collection and body composition

Tail blood glucose (Accu-Chek, Roche) and blood lactate (Lactate Plus meter, Nova Biomedical) values were determined immediately prior to the maximal exercise capacity test and immediately post, within 1 min after physical exhaustion. Blood glucose and lactate was also taken on the third session of each week both pre and post. Body composition was assessed by nuclear magnetic resonance (Echo MRI-100 Body Composition Analyzer; Echo Medical Systems, Houston, TX) was conducted at ZT17, on the days immediately prior to maximal testing.

Lumicycle analysis

For real-time bioluminescence recording, tissue explants (EDL, soleus, SCN, lungs and white fat) were washed in warm Hams F-10 1X and switched to recording medium (DMEM without phenol red (Caisson DML12-500ml) supplemented with 5 % FBS, 1mM sodium pyruvate, 1 % P/S and 0.1 mM Luciferin. SCN recording medium also contained 2 % B27 and 2 % L-glutamine). For the muscle tissues care was taken to isolate complete muscles and dissect from tendon to tendon. Whereas only a ~1cm^3 piece of lung and white fat was dissected, with care to ensure the same place and size was sampled across animals. The whole SCN slice was taken after dissection under a microscope. Tissues were cultured in vacuum-sealed 35mm dishes with a microscopy glass coverslip and placed into the Lumicycle 32 (Actimetrics, Wilmette, IL). Real-time bioluminescence recording was performed with a sampling frequency of every 10 min for at least four consecutive days as previously described (Wolff & Esser, 2012; Kemler *et al.*, 2020). The first 24 h of baseline-subtracted raw data was removed, due to the expected measurement fluctuations within the first 24 h of recording. Trimmed data was analyzed using the R based algorithm JTK Cycle (RRID:SCR_017962), to determine phase (lag), period length and amplitude (Hughes *et al.*, 2010). The Benjamini–Hochberg multiple comparison adjusted P value was used to assess the quality of the circadian curve fit. For the tissue explants, each EDL and soleus was considered a technical replicate for a given animal and chunks of lung and white fat were halved. Due to technical problems, in the SCN we were only able to include an *n* of 2 for each group.

Muscle and liver glycogen

Small (~10 mg) frozen pieces from the gastrocnemius muscle and liver were used to detect tissue glycogen content. Care was taken to ensure the same location of each tissue was sampled across replicates. Tissue samples were weighed and homogenized in 100 µl of glycogen hydrolysis buffer in a bullet blender (BBY24M, Next Advance, NY, USA). Homogenates were centrifuged at 12000 rpm for 5 mins at 4 °C. Muscle glycogen concentrations were then quantified from the supernatant using a commercially available kit (K2144, ApexBio, Houston TX, USA) according to manufacturer's instructions. Glycogen concentrations were plotted against a standard curve and background glucose was subtracted to calculate glycogen content which was expressed normalized to tissue weight.

Statistical analysis

Unless stated otherwise, data are presented as mean ± standard deviation (SD) and all statistical analyses were conducted in GraphPad Prism 9.1.2 (GraphPad Prism, RRID:SCR 002798). For multiple comparisons, data were analyzed using one or two factor analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. To assess differences between exercise training groups only, two-tailed independent student t-tests were performed to evaluate statistical differences. Real-time bioluminescence data was analyzed in the R package JTK Cycle (RRID:SCR_017962) and the Benjamini–Hochberg multiple comparison adjusted P value was used to assess the quality of the curve fit. All statistically significant thresholds were considered at the level of $p < 0.05$. All raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Morning runners exhibited enhanced adaptations compared to afternoon runners.

To evaluate the effects of time-of-day treadmill training on maximal endurance performance, we first performed a maximal endurance capacity test prior to training at two distinct times during the active period. In accordance with previous work in humans (Küüsmaa *et al.*, 2016) and mice (Ezagouri *et al.*, 2019), we established that mice tested in the afternoon (ZT22) exhibited significantly greater treadmill endurance capacities than those tested in the morning (ZT13)(Figure 1B). To allow for comparisons among the mice, we calculated treadmill work done which considers the treadmill speed, incline, duration and mouse bodyweight. We determined that the afternoon runners performed/completed 85% more treadmill work than the morning runners (2552 (260) arbitrary units [AU] vs. 1381

(354) AU)($p < 0.0001$; Figure 1B). Additionally, mice tested in the afternoon achieved an 83% farther distance ($p < 0.0001$; Figure S1A) and spent 57% longer duration on the treadmill ($p < 0.0001$; Figure S1B) their morning testing counterparts.

Ten days following pre-testing, mice were randomly assigned to complete 6 weeks of scheduled run training during the morning or afternoon. Maximum endurance capacity was tested at 3 timepoints; i) prior to the onset of training, ii) after 3 weeks of training, and iii) after 6 weeks of training. Within each time-of-training group, we found that the morning mice exhibited significant increases in endurance capacity from onset to 3 weeks ($p = 0.0400$) and 3 weeks to 6 weeks of training ($p = 0.0046$). In contrast, the afternoon training group demonstrated a significant change in endurance performance when comparing between the pre-test to 6 weeks ($p = 0.0425$), but endurance capacity at 3 weeks was not statistically different from either onset ($p = 0.2048$) or week 6 values ($p = 0.0639$)(Figure 1C). The plotted trajectory of individual mouse endurance time is provided in supplemental data (Figure S2) which suggests that rate of change in endurance capacity is greater in the morning trained compared to the afternoon trained mice.

In Figure 1D we compared maximum endurance capacity (work done) between each time-of-training group for each maximal test. After 3 weeks of training, the afternoon mice continued to perform significantly better, completing more treadmill work (47%; $p = 0.0044$) compared to those tested in the morning. However, following 6 weeks of run training, the endurance performance of mice that trained during the morning increased and was not different from those that trained during the afternoon ($p = 0.2182$)(morning runners: 3214 (384) AU vs afternoon runners: 3708 (481) AU; Figure 1D). Together, these endurance performance results indicate that mice in the morning training group exhibited a greater rate of adaptation in endurance performance compared to the mice trained in the afternoon. By week 6, morning runners exhibited no difference in treadmill performance compared to the afternoon runners.

Lastly, we wanted to examine the differences in absolute work done during training between morning runners and afternoon runners to understand how differences in the average amount of work done may have affected maximal performance (Figure 1E). We show work done on average across weeks 1 to 3 ($p < 0.0001$) and weeks 4 to 6 ($p < 0.0001$) of training was significantly greater for afternoon runners compared to morning runners. Therefore, morning runners were subject to less absolute work, despite using equal relative workloads in training.

Figure 1

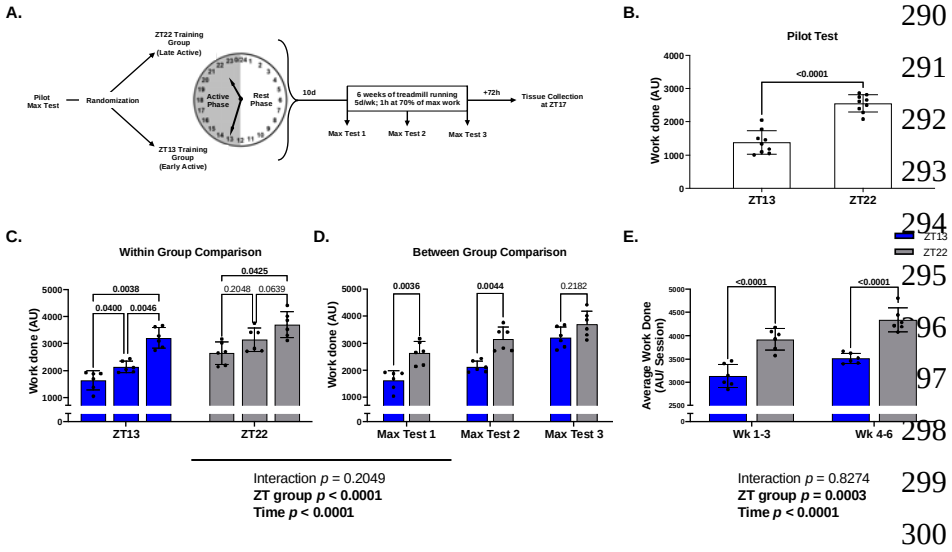


Figure 1. Time of exercise training reduces diurnal differences in exercise performance. All data are presented as MEAN (SD) with individual values plotted, and significant p-values are bolded in the figure for identification. Each maximal test was conducted at ZT13 (blue bars) for the morning runners and at ZT22 (gray bars) for the afternoon runners. A. A schematic of the experimental design is presented to show the time-line of testing, grouping, and training. B. Ten days prior to the onset of training, a pilot test was conducted to confirm if diurnal differences in maximal run performance existed for Morning Runners (n = 9) Afternoon Runners (n = 9). C and D. Maximal endurance capacity testing was conducted prior to (Morning runners: n = 6); Afternoon Runners: n = 6), 3 weeks after, and 6 weeks after onset of training. Post-Hoc comparisons of morning runners and afternoon runners are shown through within-group and between-group comparisons. E. Average training volume of Morning and afternoon runners were also compared across weeks 1-3 and weeks 4-6.

Blood glucose, blood lactate response to exercise and tissue glycogen content with training show no time-of-day differences.

To corroborate our time-of-day exercise capacity outcomes, well-established markers of acute exercise and training responses were evaluated. To interrogate the acute response during maximal endurance exercise we measured blood glucose and blood lactate immediately before and after each maximum endurance test. We observed significantly higher measures of blood glucose ($p < 0.05$) and blood lactate ($p < 0.05$) immediately after each maximum capacity test (Figure 2A & 2B). However, there was no difference between morning and afternoon runners for either measure, and there was no change with training.

Thus, the acute glucose/lactate response does not differ between time-of-training groups and does not show any pattern that associates with differences during any endurance capacity tests.

It is well know that endurance training results in increased storage of glycogen in both muscle and liver (18, 34, 35). Thus, we tested whether there were any differences in tissue glycogen abundance that could be a potential reason for our time-of-day-specific training outcomes. After 6 weeks of training, we found skeletal muscle ($p > 0.9999$) and liver ($p = 0.1504$) glycogen content were not different between morning and afternoon runners. In addition, we did not observe differences in glycogen abundance in the liver of morning (12.4 (4.0) μg/mg)($p = 0.0662$) or afternoon (9.5 (1.5) μg/mg tissue)($p = 0.8895$) runners, compared to sedentary controls (8.8 (1.0) μg/mg tissue); Figure 2C). However, skeletal muscle glycogen was higher in morning (1.5 (0.2) μg/mg)($p < 0.0001$) and afternoon (1.5 (0.2) μg/mg)($p < 0.0001$) runners compared to sedentary controls (0.6±0.1 μg/mg)(Figure 2D). Thus, we did not find any time-of-day training effects on the magnitude of glycogen storage.

Figure 2

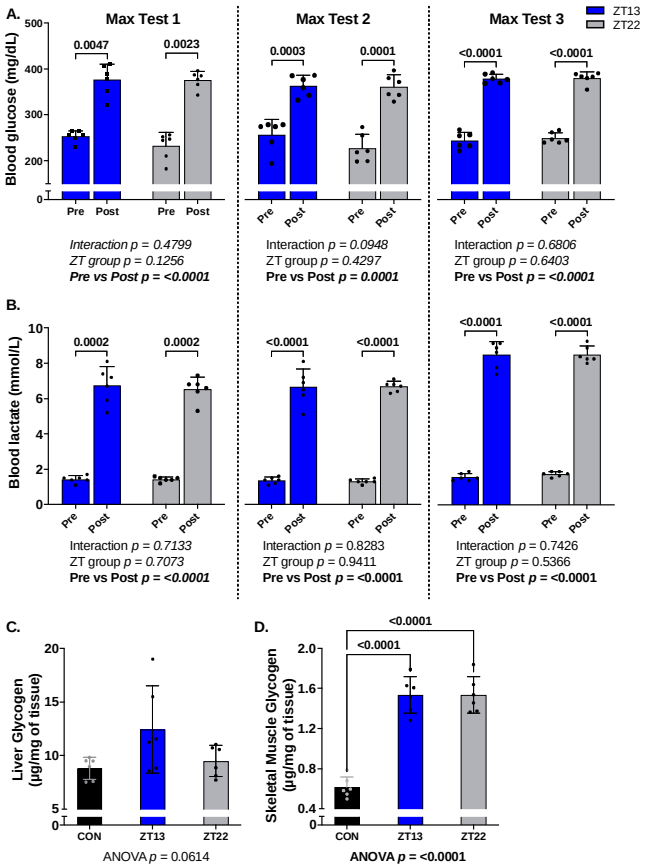


Figure 2. Maximal capacity test blood markers and basal tissue glycogen content. All data are presented as MEAN (SD) with individual values plotted, and significant p-values are bolded in the figure for identification. Each maximal test in A and B was conducted at ZT13 for the morning runners (blue bars)(n =6) and at ZT22 for the afternoon runners (gray bars)(n = 6). **A and B.** Acute blood markers of glucose and lactate were collected both pre and post each maximal testing bout. **C and D.** Tissue glycogen content for liver (µg/mg of tissue) and skeletal muscle (µg/mg of tissue) was collected 72h after completion of the final endurance testing session. Figures C and D are represented with black bars for control mice (n = 6), blue bars for morning runners (n = 6), and gray bars for afternoon runners (n = 6).

Increased Daily Cage Activity and Body Composition during Exercise Training

We next asked if there were differences in the daily cage activity in the mice in the morning or afternoon training groups. For these experiments we used infrared motion sensors in the home cage to track 24hr movement around the cage. We found that mice from both training groups exhibited more movement in their home cages compared to the control mice throughout the 6 weeks of training (morning runners: Week 1, Week 3, and Week 6 p <

0.0001; afternoon runners: Week 1 and Week 3 $p < 0.001$, Week 6 $p = 0.0001$)(Figure 3A). We noted that the activity of the mice was largely limited to the normal active period (dark phase) with no indication of altered activity in the light or sleep phase of the day (data not shown or in supplement). We did detect cage activity differences between the morning and afternoon training groups with the afternoon runners showing more daily cage activity in the first week ($p = 0.0017$), while the morning runners had more cage activity in the last week of training ($p < 0.0001$). It is important to note that these measures are reflecting behavioral changes based on movement in the cage but cannot extracted distance traveled.

We also sought to investigate if time-of-training elicited differential changes in body mass and/or body composition and if there were any alterations that associated with the differences in work done during treadmill running. We measured weekly food consumption in all mice and found there were no significant differences in the amount of food consumed across all 3 groups (Figure S3). We tracked body weight and body composition at weeks 1, 3 and 6 following training in all groups. There were no significant differences between sedentary control, morning runners, or afternoon runners in body mass (Figure 3B) or percent lean mass (Figure 3C). However, percent fat mass was significantly reduced at 6 weeks in both morning ($p = 0.0010$) and afternoon runners ($p = 0.0014$) compared to sedentary controls (Figure 3D). These results indicate that time-of-day training over 6-week did not significantly alter body mass or lean mass but did reduce fat mass in training groups. Because there are no differences between time-of-day training groups, we suggest changes in fat mass do not correlate with the time of training performance differences.

Figure 3

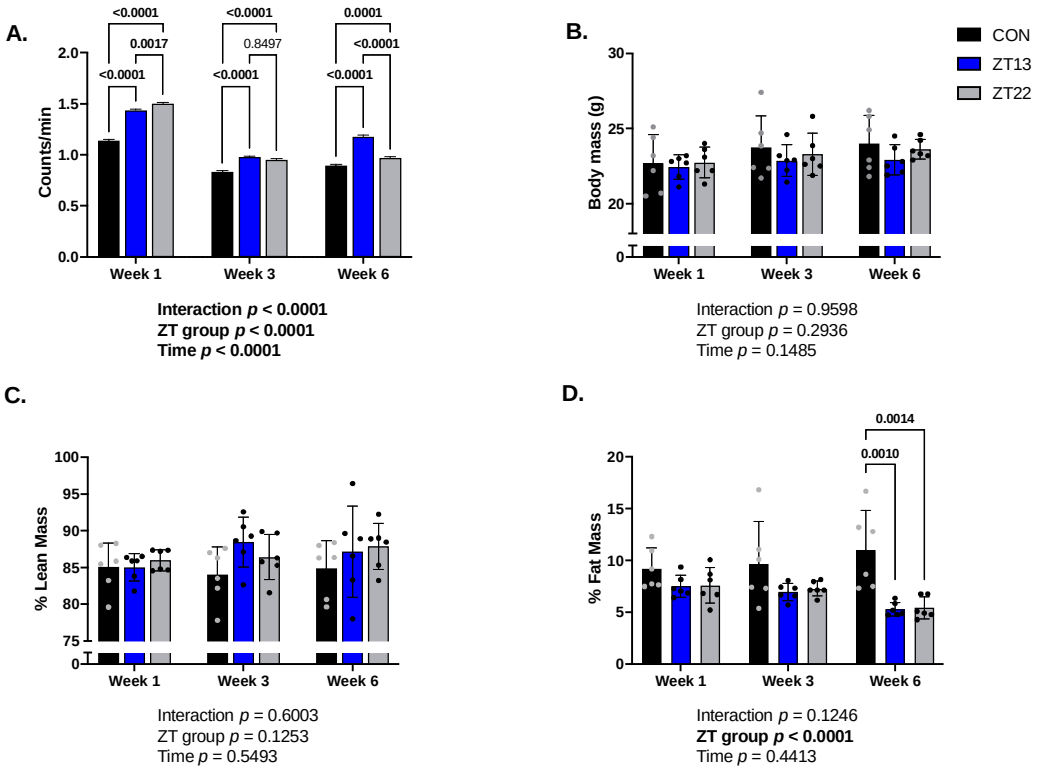


Figure 3. PER2::LUC mice cage activity and body composition profile.

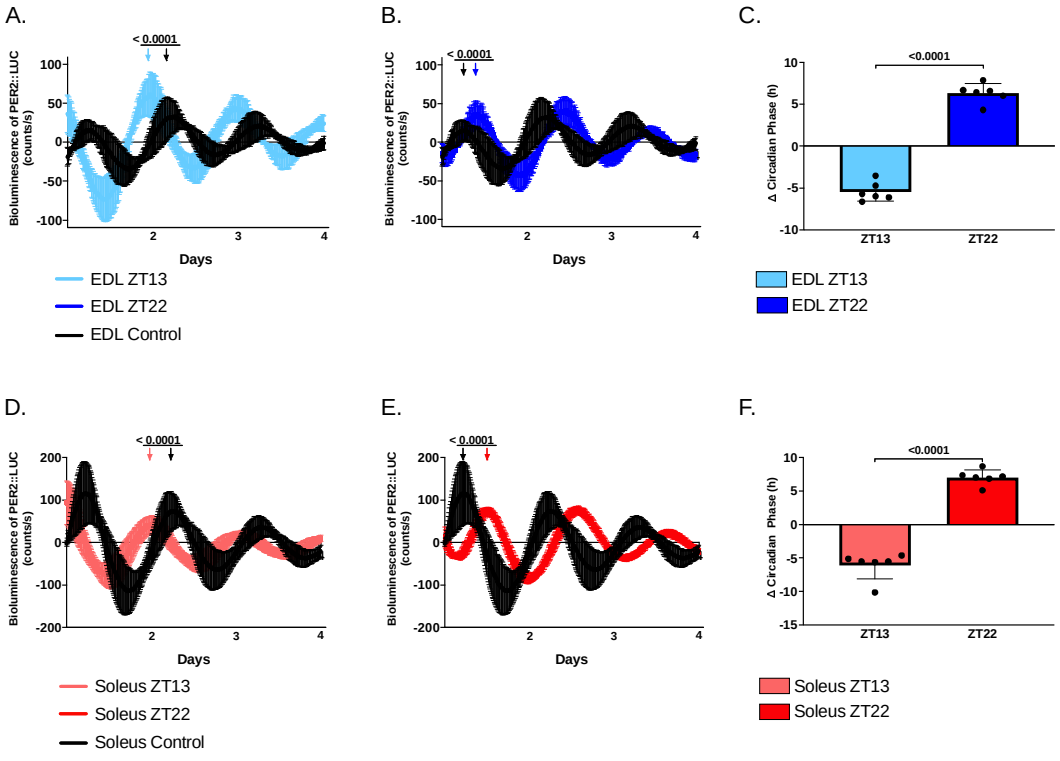
Unless stated otherwise, data are displayed as MEAN (SD) with individual values plotted, and significant p-values are bolded in the figure for identification. Mice were individually housed for the duration of the time-of-day training program, and all animals were evaluated at weeks 1, 3, and 6 for cage activity and body composition. The sedentary control group is shown in black (n = 6), the ZT13 mass) are displayed to visually represent group comparisons over time.

Tissue-Specific Circadian Phase Responses to Morning and Afternoon Exercise Training.

We used tissues from the circadian reporter mice, PER2::LUC, to ask if exercise training at different times within the active period would lead to sustained changes in the phase of the skeletal muscle clock. For these experiments we collected the tissues from the mice 3 days following the last bout of exercise to avoid any potential acute effects of exercise. We collected both soleus and EDL muscles from mice to address whether there were any fiber type or muscle recruitment specific effects on the phase of the muscle clocks following morning or afternoon active period run training. Using real-time bioluminescence recording, we determined that the clocks in the EDL muscles of morning runners exhibited a phase advance of ~ 5.5 hours compared to the clocks in the control sedentary EDL muscles (p

398 < 0.0001)(Figure 4A & 4C). In contrast, the clocks in the EDL muscles of afternoon runners
399 experienced a ~6.3-hour phase delay ($p < 0.0001$)(Figure 4B & 4C). Analysis of the data
400 from soleus muscles found that the muscle clock phase changes were similar in magnitude to
401 the EDL, with the soleus muscles of morning runners exhibiting a phase advance of ~6.1
402 hours ($p < 0.0001$)(Figure 4D & 4F), and the muscle clocks from the afternoon runners
403 experienced a phase delay of ~ 7 hours ($p < 0.0001$)(Figure 4E & 4F). While there were
404 significant changes in phase, there were no significant changes in the amplitude of the
405 PER2::LUC rhythms between time-of-day exercise training groups (Figure S4A-E). These
406 findings demonstrate that run training in the morning or afternoon results in robust (>5hr) and
407 stable shifts in the clocks within the soleus and EDL muscles. These findings are consistent
408 with observations of acute time-of-day exercise on the muscle clocks and reinforce the
409 concept that exercise can function as a non-photic time cue, or zeitgeber, *in vivo*, and this is
410 irrespective of fiber type and muscle recruitment or loading patterns.

411 **Figure 4**



412

413 **Figure 4. Effects of 6 weeks exercise training on circadian phase of the skeletal muscle**
414 **clock.**

415 All data is represented as mean (SD) with individual values plotted for C and F, and
416 significant p-values are bolded in the figure for identification. All animals in the sedentary

control group (*n* = 6), ZT13 (*n* = 6) and ZT22 (*n* = 6) exercise training groups were killed 72 hours after their last bout of exercise, and tissues were placed in a lumicycle for culture at ZT17. Real-time bioluminescent tracing (baseline subtracted) of PER2::LUC activity is shown over 3 consecutive days for the explanted muscles. All data shows the sedentary control group as black. **A and B.** The extensor digitorum longus (EDL) muscle are shown in light blue for morning runners (ZT13) and in dark blue for the afternoon runners (ZT22)**,** with the change in EDL phase from the control mice represented in **C**. **D and E.** The soleus is in light red for ZT13 and in dark red for ZT22, with the change in soleus phase from control mice shown in **F**. Arrows shown in **A**, **B**, **D**, and **E** indicate the timing of peak luminescence. For **C** and **F**, a decrease in phase indicates a phase advance, and an increase in phase represents a phase delay.

While skeletal muscle is the common target for exercise studies, *in vivo* exercise training leads to adaptations across different organ systems (MoTrPAC Study Group *et al.*, 2023). We isolated the central clock (SCN), and lung and white adipose tissues to address potential shifts in non-muscle tissue clocks. Analysis of PER2::LUC bioluminescence of lung tissue (Figure 5A & 5D) from morning and afternoon exercised mice found that there was no significant phase shift within the lung tissue of morning runners, but the lung tissue of afternoon runners exhibited phase delays by an average of 6.1 hours ($p = 0.0004$)(Figure 5A & 5B). Like lung tissue, the WAT clocks from morning runners showed no significant changes in the timing of their circadian phase, however, the WAT of afternoon runners showed a ~5.4-hour phase delay (Figure 5D & 5E). Lastly, we examined the effect of time of exercise training on the phase of the central clock within the suprachiasmatic nuclei of the hypothalamus in a subset of animals. Consistent with what has been reported for run training (Wolff & Esser, 2012), we did not detect any exercise training-induced phase advances or delays in the SCN (Figure S5A-G). These results illustrate the complexity of systemic signals that regulate the phase of the peripheral tissue clocks. Unlike muscle neither WAT or lung clocks exhibited a phase advance with morning running, but like muscle clocks, the clocks withing WAT and lungs shared the significant phase delays with afternoon running. Much more research is needed to identify the upstream time cues and molecular mechanisms modifying non-muscle clock shifts with exercise training.

Figure 5

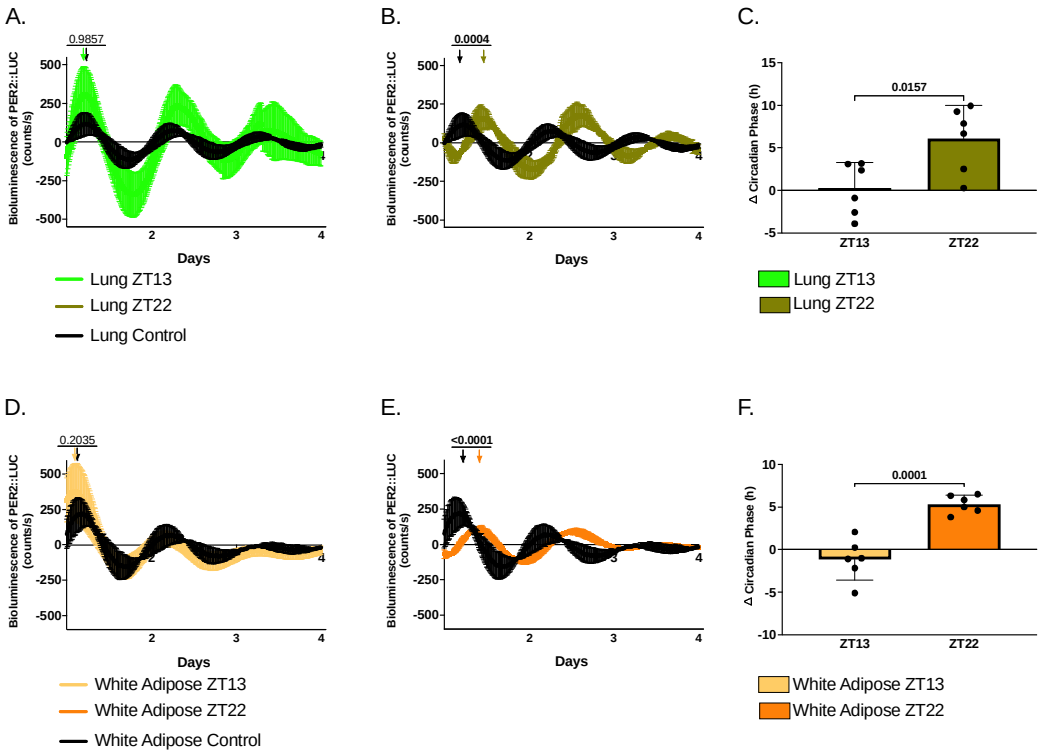


Figure 5. Effects of 6 weeks exercise training on circadian phase of the intrinsic clock for lung and white fat.

All data is represented as mean (SD) with individual values plotted for C and F, and significant p-values are bolded in the figure for identification. All animals in the sedentary control group (*n* = 6), ZT13 (*n* = 6) and ZT22 (*n* = 6) exercise training groups were killed 72 hours after their last bout of exercise, and tissues were placed in a lumicycle for culture at ZT17. Real-time bioluminescent tracing (baseline subtracted) of PER2::LUC activity is shown over 3 consecutive days for the explanted tissues. All data shows the sedentary control group in black. **A and B.** The lung tissue is shown in light green for the morning runners (ZT13) and in darker green for the afternoon runners (ZT22), with the lung phase difference compared to sedentary controls shown in **C**. **D and E.** White adipose tissue is shown in light orange for ZT13 and in darker orange for ZT22**,** with the white adipose phase difference compared to sedentary controls shown in **F**. Arrows shown in **A**, **B**, **D**, and **E** indicate the timing of peak luminescence. For **(C)** and **(F)**, a decrease in phase indicates a phase advance, and an increase in phase represents a phase delay.

Discussion

Here, we demonstrate that 6 weeks of treadmill run training, in female mice, during the morning leads to enhanced endurance performance compared to training in the afternoon,

despite both groups training at the same relative intensity. The enhanced performance in morning runners cannot be explained by measured metabolic markers, differences in body mass or body composition. However, morning runner performance was associated with a large ~5 hour phase advance of the skeletal muscle clock. Thus, we suggest that the enhanced performance outcomes of the morning time-of-training group is linked to the large phase advance in the skeletal muscle clock, which potentially serves to better align substrate metabolism with the time of performance.

Endurance exercise capacity in mice has been shown by our lab and others to be lower during the morning hours of the active phase compared to the afternoon hours (Wolff & Esser, 2012; Ezagouri *et al.*, 2019; Sato *et al.*, 2019; Adamovich *et al.*, 2021*b*, 2021*a*; Maier *et al.*, 2022). In this study we reveal that endurance exercise training during the morning overcomes the established time-of-day difference in maximal endurance capacity. However, this was not evident following 3 weeks of training, which is consistent with the findings of Adamovich and colleagues that showed time-of-day endurance performance was still different after 2 weeks of run training (Adamovich *et al.*, 2021*b*). We found that differential performance between morning and afternoon runners required 6 weeks of treadmill training to be overcome. This is consistent with findings from Souissi and colleagues that demonstrated that 6 weeks of resistance training in humans was sufficient to overcome time-of-day maximal strength differences, with the effects persisting at 2 weeks post training (21). Thus, the data show time-of-day performance differences can be overcome, but it requires several weeks of regular training in the morning to be realized.

As an important design feature of this study, we carefully controlled the training workloads so that each group trained at the same relative workload (70% of max) based on individualized maximal run performance. Relative workloads are commonly used to normalize the intensity of exercise across individuals with different maximal capabilities in order to improve the specificity of training (Hawley, 2002; Nordsborg *et al.*, 2010; Mann *et al.*, 2013, 2014; Egan & Sharples, 2023). For example, like our study with mice, Souissi and colleagues, also applied relative training loads, as percentages of time-of-day maximal knee extensor strength, for each participant, and updated after mid-study re-testing. It is important to note that since we standardized the training based on relative workloads, this means that the morning runners achieved an enhanced rate of endurance performance adaptation despite training at a lower absolute treadmill workload compared to the afternoon runners. This outcome was surprising as we saw no between-group differences in other factors that influence treadmill run performance like body weight (Winter, 1979; Avila *et al.*, 2017), lean

body mass (%) (Maciejczyk *et al.*, 2014), and glycogen abundance in liver and muscle (López-Soldado *et al.*, 2021). This suggests that the mechanisms underpinning the enhanced performance among morning runners were likely unique to time-of-day training.

Prior studies using genetic mouse models have determined that time-of-day endurance performance differences required the circadian clock system. In this study we asked whether time-of-day training was sufficient to modify the steady-state settings of the muscle circadian clock. Using real time bioluminescence recording from tissues of the control and trained PER2::LUC mice we determined that the morning runners exhibited a ~5-hour phase advance in both the EDL and the soleus muscles but no change in lung or white fat clocks. This is contrary to the results from Adamovich and Colleagues' 2-week training study in which there was no significant change in the phase of the muscle clock with morning training (Adamovich *et al.*, 2021*a*). Since all tissues in this study were collected 72 hours following the final testing bout, these changes in clock settings represent a steady state shift rather than an acute exercise response. Lastly, inclusion of both the soleus and EDL muscles in this study allow us to conclude that the exercise induced clock shifts are not specific to muscle innervation, fiber type composition, or muscle motor pattern function, due to the identical phase shifts of the clocks in these two diverse muscles.

Previous literature using loss of function genetic mouse models have demonstrated that the circadian clock mechanism is necessary for the time-of-day exercise performance. Additional analyses suggested this was due to glycogen storage by the liver associated with feeding as contributing factors (Ezagouri *et al.*, 2019; Adamovich *et al.*, 2021*a*). Our data provide new insight into these time-of-day exercise performance differences with evidence that diurnal differences in exercise capacity may be directly influenced by the skeletal muscle circadian clocks. Potential insight into the mechanism may come from data from van Moorsel et al (van Moorsel *et al.*, 2016), demonstrating that human muscle mitochondrial oxidative capacity exhibited a time-of-day pattern with highest capacity in the late afternoon. These observations were followed by data from Gemmink and colleagues (Gemmink *et al.*, 2023), demonstrating time-of-day fluctuations in mitochondrial morphology that mirrors oxidative function. Additional papers have highlighted various aspects of mitochondrial structure and function with muscle circadian clocks (Liu *et al.*, 2007; Jordan & Lamia, 2013; Jacobi *et al.*, 2015; Sardon Puig *et al.*, 2018). These studies indicate that circadian clock output in muscle contributes to the daily variations in mitochondrial oxidative capacity. From this, we propose that the ~5h advance in the muscle clock in morning runners temporally shifts the peak of clock output including genes contributing to mitochondrial oxidative capacity. With this large

shift in clock phase, the peak mitochondrial function will better align with time on the treadmill. Said differently, we propose that the enhanced adaptations of the morning runners are the result of a phase advancement in mitochondrial oxidative capacity driven by shifts in the skeletal muscle clock. This hypothesis is substantiated by recent findings from Xin and colleagues (Xin *et al.*, 2023*b*) which demonstrated that using time restricted feeding as a method to advance the muscle clocks, is sufficient to improve rest phase maximal endurance capacity, even in the absence of exercise training (Wolff & Esser, 2012). They found that the increased performance was associated with an alignment of the expression profile of the oxidative metabolism gene network in muscle to time-of-feeding. Importantly, this effect was not observed in mice without a functional whole-body or muscle-specific clock mechanism, illustrating that the muscle clock directed the changes in endurance performance observed with rest-phase-restricted feeding. The implications of this body of work suggests that when implementing an exercise training regimen, it will be important to consider time-of-day to support alignment of the muscle clock and metabolic profile to the time of performance.

In conclusion, we report that six weeks of treadmill exercise training induces an enhanced endurance performance adaptation in the morning (ZT13) compared to afternoon (ZT22) training groups. This enhanced adaptation in the morning training group is associated with a significant ~5hr advance in the muscle clock phase with no changes in the central clock or white fat and lung clocks. Studies have demonstrated important links between the muscle clock and daily modulations in metabolism, in particular oxidative metabolism. Thus, we propose that this enhanced adaptation to morning training is due to shifts in phase of the muscle clock to better align metabolic capacity of the muscle with exercise demands. While exercise is known to exert a myriad of positive health effects, the concept that these benefits may be conferred, in part, through changes in circadian rhythmicity, remains under studied. More work must be done to truly link exercise induced changes in muscle circadian phase, endurance performance, and mitochondrial oxidative capacity because such a relationship has sweeping implications for athletic performance, experimental exercise-research design, as well as restoring functional deficits in individuals with circadian disruption (e.g., type 2 diabetes and aging (Broussard *et al.*, 2012; Silva *et al.*, 2021; Savikj *et al.*, 2022).

Additional information

Competing interests

Authors have no competing interests to declare.

Author contributions

S.J.H. was responsible for conception, coordination, and completion of experimentation. The manuscript was primarily drafted by C.L.S., S.J.H., and K.A.E. Analysis and interpretation was conducted by all authors. Critical review of all intellectual content within the manuscript was provided by C.A.W., M.R.V. The final manuscript was read and approved by all authors, and each agrees to be accountable for all aspects of the work. All authors have worked to ensure the accuracy and integrity of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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